genesis procedures. Cells were metabolically labelled 3 days after transfection with [^{32}P] phosphate for 3 h, treated with the indicated factors (5 nM Bmp-2, Genetics Institute; 18 nM EGF, R&D Systems; 1.1 nM HGF, R&D Systems) and lysed. Where indicated, cells were treated with the MEK1 inhibitor PD98059 (100 μM, New England Biolabs) or the PI(3) kinase inhibitor wortmannin (0.1 μM, Calbiochem) for 1 h before addition of growth factor. Serum starvation was for 14 h before metabolic labelling. After cell lysis, Flag–Smad1 was precipitated with monoclonal anti-Flag antibody (M2, Kodak Scientific) and proteins were resolved by SDS–PAGE and visualized by autoradiography. Parallel cultures were treated equivalently, lysed and subjected to western immunoblotting with anti-Flag antibody M2 and by chemiluminescence (ECL, Amersham).

Kinase assays. Smad1 and the Smad1 linker domain (amino acids 146–264) were subcloned into a pET expression vector (Novagen) encoding an N-terminal hexahistidine tag. Bacterial expression and purification of recombinant proteins were performed as described[6]. Smad1 proteins were preincubated (30 min at 4 °C) with recombinant, activated BMPR-IB(Q203D) cytoplasmic domain (amino acids 150–502)[6] or with recombinant, activated Erk2 MAP kinase (New England Biolabs) in a buffer containing 50 mM Tris-HCl, pH 7.3, 100 mM NaCl, 10 mM $MnCl_2$, 10% (v/v) glycerol, 5 mM dithiothreitol, and 0.05% (v/v) Triton X-100. Upon [γ^{32}P]ATP addition, reactions were performed at 28 °C for 20 min. Reactions were stopped by addition of a buffer containing 6 M guanidinium-HCl, and Smad1 was recovered with Ni-NTA agarose.

Phosphopeptide analysis. Smad1 protein was immunoprecipitated from transfected, [^{32}P]phosphate-labelled R-1B/L17 cells, separated by SDS–PAGE, and transferred to nitrocellulose. Membrane pieces containing Smad1 were incubated with trypsin, chymotrypsin and endopeptidase Lys-C (Promega, Boehringer-Mannheim, and Worthington, respectively) in 50 mM ammonium bicarbonate at 37 °C for 3 h. Digests were resolved on 16.5% Tris-Trycine gels[32] and visualized by autoradiography. M_r markers were ^{14}C-methylated peptide fragments (Sigma).

SMAD association assay. In SMAD association experiments[6], COS-1 cells co-transfected with haemagglutinin-tagged Smad4, Flag-tagged Smad1 and BMPR-IB(Q203D) were treated with EGF (18 nM) for 15 min before Bmp-2 addition. Cells were lysed[6] and portions used in anti-Flag immunoblotting assays to control the Smad1 expression level. The remainder of the cell lysates were immunoprecipitated with anti-Flag antibody. Immunoprecipitates were washed and subjected to anti-haemagglutinin immunoblotting.

Luciferase assays. Luciferase assays were performed essentially as described[7]. In brief, R-1B/L17 cells were transiently cotransfected with a Gal4-luciferase reporter construct[33] (2 μg) and the indicated Gal4 or Gal4-Smad1 expression constructs (0.5 μg). Cells were serum starved for 12 h before treatment with Bmp-2 (5 nM) and/or EGF (18 nM), and Luciferase assays were performed 18 h after addition of growth factor.

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Smad6 inhibits signalling by the TGF-β superfamily

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SMAD proteins[1] have been identified as signalling mediators of the TGF-β superfamily, which is involved in a range of biological activities including cell growth, morphogenesis, development and immune responses[2,3]. Smad1, Smad2, Smad3 and Smad5 are ligand-specific: Smad1 and Smad5 transduce signals from bone morphogenetic proteins[4–7], and Smad2 and Smad3 mediate signalling by TGF-β and activin[8,9], whereas Smad4 acts as a common signalling component[10]. For example, Smad2 is phosphorylated by the TGF-β type I receptor upon ligand binding, forms a heteromer with Smad4, and then translocates into the nucleus where it activates transcription[10,11]. Here we report the isolation of Smad6 in the mouse. Smad6 is quite different in structure from the other SMAD proteins, and forms stable associations with type I receptors. Smad6 interferes with the phosphorylation of Smad2 and the

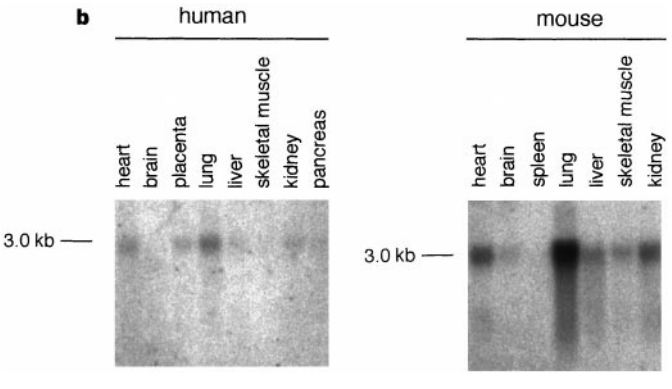

Figure 1 Protein sequence alignment and tissue distribution of mouse Smad6. **a**, The deduced amino-acid sequence of Smad6 is aligned with other mammalian SMAD proteins. Identical residues are boxed. The N-terminal two-thirds of mouse Smad6 shows little similarity with the other SMADs. **b**, Human (left) and mouse (right) tissue blots were hybridized with the human EST clone 429356 as a probe. A transcript of 3.0 kb is detected, with the highest expression in lung in both blots.

subsequent heteromerization with Smad4, but does not inhibit the activity of Smad3. Smad6 also inhibits the phosphorylation of Smad1 that is induced by the bone morphogenetic protein type IB receptor. These data indicate that signals of the TGF-β superfamily are regulated both positively and negatively by members of the SMAD family.

We have identified the complete coding sequence of murine Smad6 by screening a mouse lung cDNA library with an expressed-sequence tag (EST) clone (429356) as a probe. Partial sequence information and chromosome localization of the human homologue of this gene has been reported[12] (accession no. U59914). Smad6 is a protein of 495 amino acids with a predicted relative molecular mass of 53,700 (M_r 53.7K) (Fig. 1a). All of the SMAD proteins, except the human homologue, including those of *Drosophila* and *Caenorhabditis elegans*, have conserved amino- and carboxy-terminal regions (MH1 and MH2, respectively) separated by a proline-rich linker region of variable length and sequence, although Smad4 has a unique insert in its MH2 region[1]. The C-terminal one-third of Smad6 shares the conserved sequence with the MH2 regions of the other SMAD proteins, but its N-terminal region shows a striking difference from the conserved MH1 sequence (Fig. 1a), suggesting that it has a different function. Northern blot analysis of various human and mouse tissues revealed relatively ubiquitous expression of the mRNA species of 3.0 kilobases (kb), with lung having the highest expression (Fig. 1b).

Members of the transforming growth factor (TGF)-β superfamily exert their diverse effects by binding to two types of receptor with serine/threonine kinase activity[3]. The ligand first binds to the type II receptor, which consequently activates the type I receptor by direct phosphorylation. The activated type I receptor then phosphorylates ligand-specific SMAD proteins, such as Smad1, Smad2 and Smad3 (refs 1, 9–11). The association of Smad2 with the TGF-β type I receptor (TβR-I) requires activation of TβR-I by the type II receptor (TβR-II)[11]. Smad2, however, interacts with TβR-I only transiently under physiological conditions, as Smad2 is released from TβR-I after phosphorylation by the receptor. The interaction of Smad2 with TβR-I has thus been observed only when the kinase-defective form of TβR-I is used[11]. It should be noted that Smad4 does not associate with the receptors[9].

We examined the interaction of Smad6 with the type I receptors in affinity crosslinking assays. Smad6 bound to the TGF-β receptor complexes, as indicated by the coprecipitation of the receptor complexes with Smad6 (Fig. 2a). As with Smad2, the binding of Smad6 to TβR-I required the kinase activity of TβR-II (Fig. 2a), although Smad6 stably bound wild-type TβR-I. Similar results were obtained with the activin type IB receptor (ActR-IB) (Fig. 2b) and the bone morphogenetic protein (BMP) type IB receptor (BMPR-IB) (Fig. 2c), for which Smad6 bound to both wild-type and kinase-defective type I receptors. These results suggest that Smad6 binds to the type I receptors in a ligand-dependent manner but has a function different from that of the other SMAD proteins.

We investigated the functional interaction of Smad6 with other SMAD proteins. Smad2 is phosphorylated at its C-terminal end by activated TβR-I (ref. 11). The phosphorylation is essential for the downstream signalling events that culminate in transcriptional activation of the target genes, as disruption of the phosphorylation sites abolished responses induced by TGF-β[11]. We then examined the effect of Smad6 on the phosphorylation of Smad2 (Fig. 3b). Phosphorylation of Smad2 induced by constitutively active TβR-I was suppressed by Smad6 (39% reduction, as normalized for the ^{35}S-labelled band), whereas Smad2 did not affect the constitutive phosphorylation of Smad6 (Fig. 3a). Smad3 and Smad2 have 91% identity in their amino-acid sequence and have been shown to mediate TGF-β signals[8,9], although we know of no functional differences between the two molecules. Smad6 enhanced receptor-induced phosphorylation of Smad3 (Fig. 3c), suggesting that Smad6 has different effects on these closely related molecules. We then investigated the effect of Smad6 on Smad1 phosphorylation (Fig. 3d). Smad1 was phosphorylated by both the constitutively active BMPR-IA and BMPR-IB. Smad6 efficiently inhibited phosphorylation induced by the BMPR-IA (60% reduction) but not by BMPR-IB. These results suggest that Smad6 acts as an inhibitor towards certain members of the SMAD family.

Smad2 forms a heteromer with Smad4 upon phosphorylation by

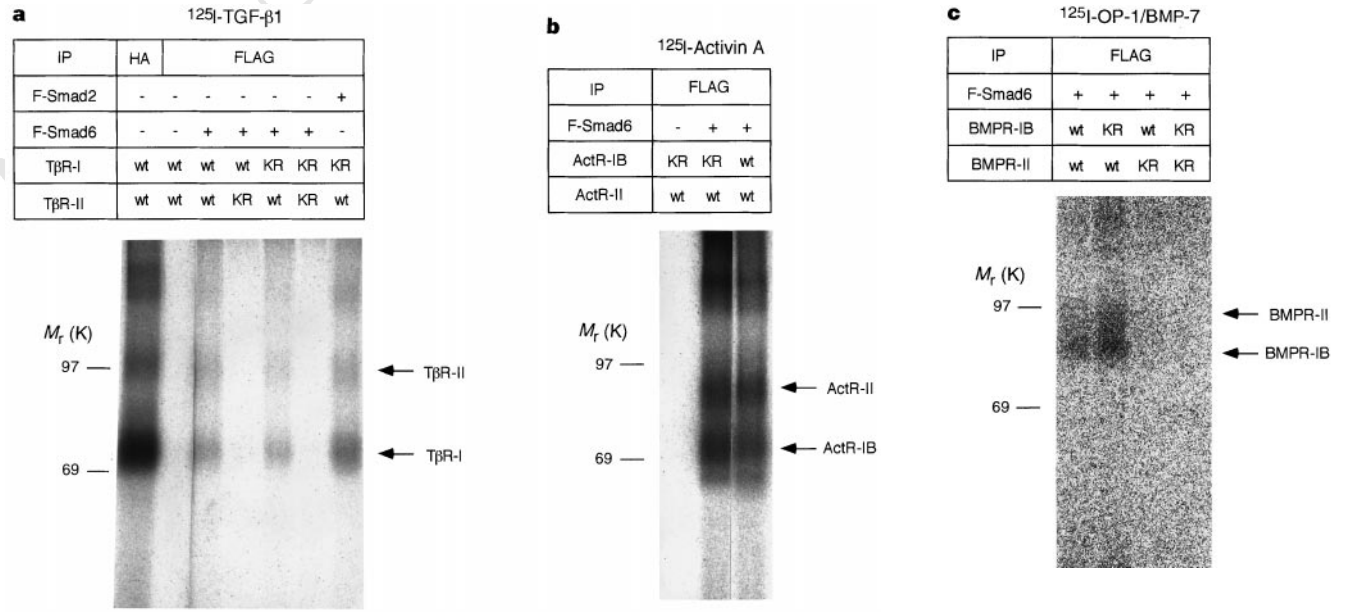

Figure 2 Binding of Smad6 to the type I receptors. **a**, COS-7 cells were transfected with Flag-tagged Smad6 (F-Smad6) or Flag-tagged Smad2 (F-Smad2) in combination with the wild-type (wt) or kinase-defective (KR) haemagglutinin (HA)-tagged TβR-I and hexahistidine-tagged TβR-II. Cells were affinity-labelled with ^{125}I-TGF-β1 and lysates were immunoprecipitated (IP) with anti-HA antibody or anti-FLAG M2 antibody. Immune complexes were subjected to SDS–PAGE and autoradiography. Smad6 bound to both wild-type and kinase-defective TβR-I, depending on the kinase activity of TβR-II. In contrast, Smad2 bound to kinase-defective TβR-I but not the wild-type TβR-I (data not shown). **b**, A similar experiment was done with ^{125}I-activin A. Smad6 associated with both wild-type and kinase-defective ActR-IB. **c**, An experiment using ^{125}I-OP-1/Bmp-7 was performed. Smad6 bound to BMPR-IB only when activated by BMPR-II.

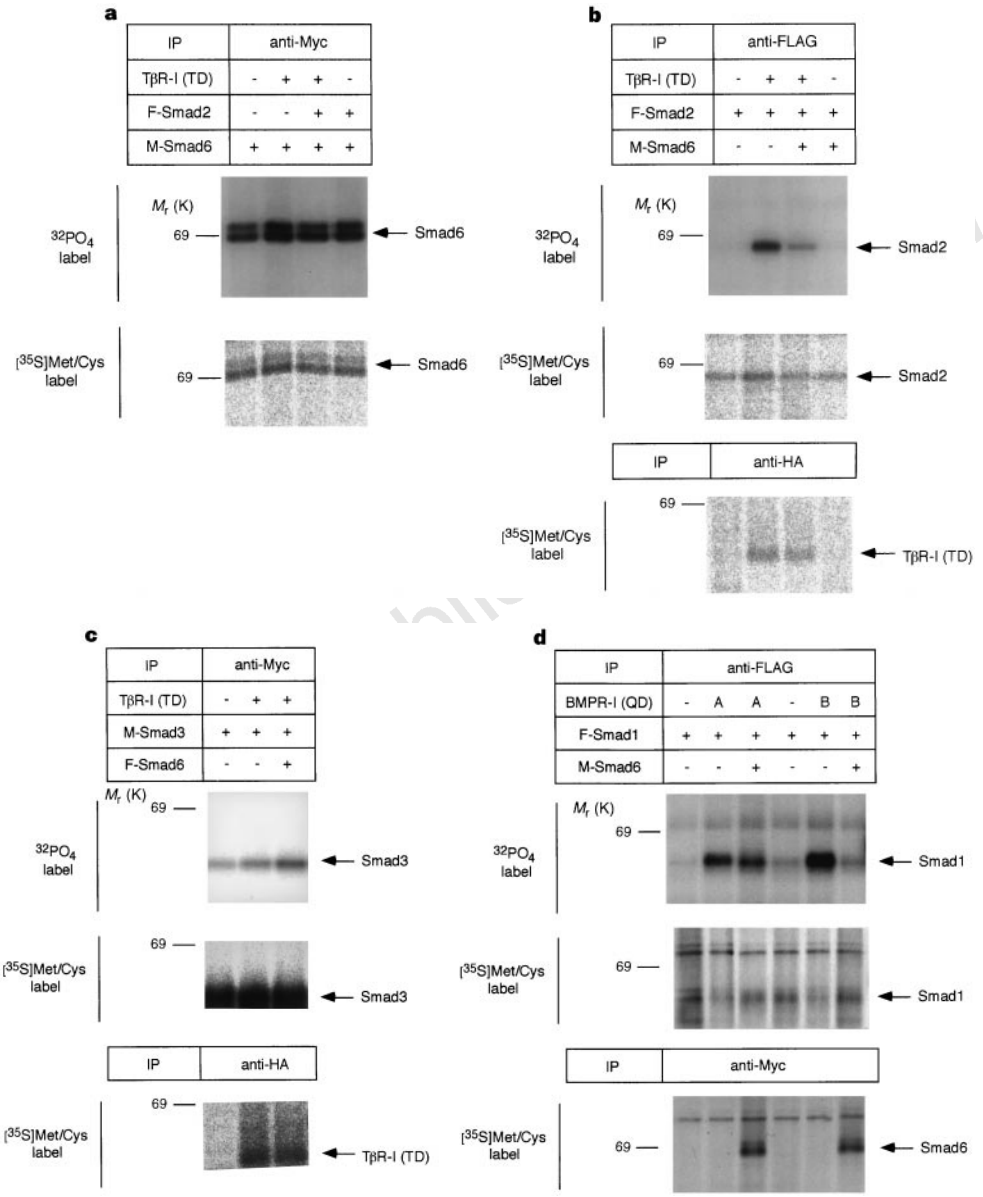

Figure 3 Effect of Smad6 on the phosphorylation of Smad2, Smad3 or Smad1 by the constitutively active type I receptors. **a**, COS-7 cells were transiently transfected with constitutively active (TD) TβR-I, Flag–Smad2 (F-Smad2), and/or Myc–Smad6 (M-Smad6). Cells were labelled with [^{32}P]orthophosphate and lysates subjected to immunoprecipitation (IP) with anti-Myc antibody. Phosphorylated Smad6 was detected by SDS–PAGE and autoradiography. Doublet bands of phosphorylated Smad6 were detected. Neither TβR-I (TD) or Flag–Smad2 affected the phosphorylation. **b**, Cell lysates were immunoprecipitated with anti-Flag antibody to detect Smad2 phosphorylation. Expression of Smad6 suppressed phosphorylation of Smad2 induced by TβR-I (TD). Expression levels of Smad6 (**a**) and Smad2 and TβR-I (TD) (**b**) were monitored by labelling the cells with [^{35}S]methionine/cysteine. **c**, A similar experiment was done with Smad3. Smad6 did not suppress receptor-induced phosphorylation of Smad3. **d**, Smad1 was phosphorylated by constitutively active (QD) BMPR-IA as well as by BMPR-IB. Smad6 inhibited phosphorylation of Smad1 that was induced by BMPR-IB, but not by BMPR-IA.

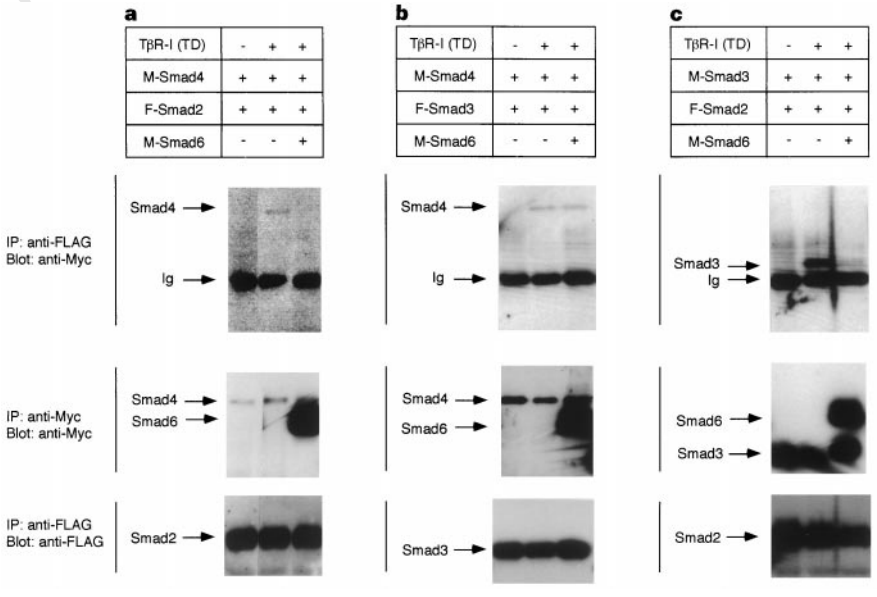

Figure 4 Effect of Smad6 on the heteromerization of Smad2, Smad3 and Smad4. **a**, COS-7 cells were transfected with the indicated combination of plasmids and subjected to immunoprecipitation (IP) followed by western blot detection. Myc–Smad4 (M-Smad4) and Flag–Smad2 (F-Smad2) interacted in the presence of TβR-I (TD), but this was abolished by the expression of Myc–Smad6 (top). Expression levels of Smad2 (bottom), Smad4 (middle), and Smad6 (middle) were monitored. Note that Smad6 did not interact with Smad2 under these conditions (top). **b**, Smad6 did not affect receptor-induced association of Smad3 and Smad4. Smad4 coprecipitated with Smad3 both with and without Smad6 (top). **c**, TβR-I (TD) induced association of Smad2 and Smad3, but this was abolished by Smad6. Smad3 coprecipitation with Smad2 disappeared in the presence of Smad6 (top). Ig, immunoglobulin.

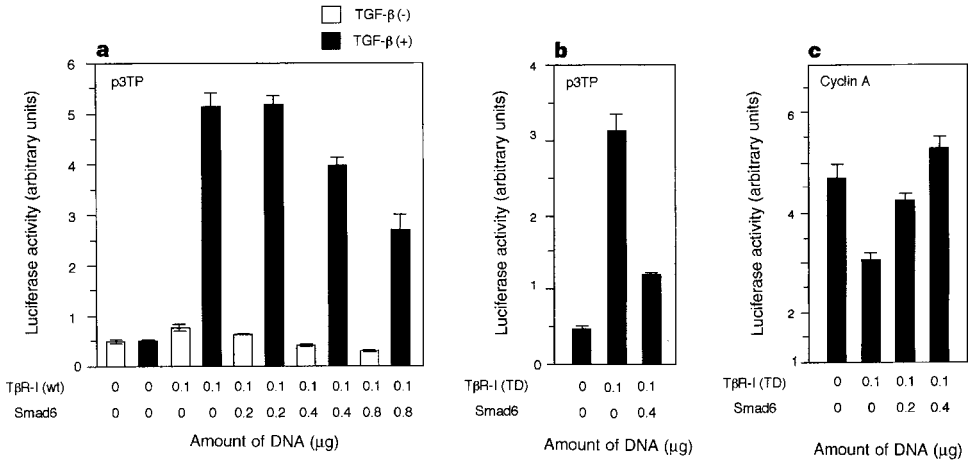

Figure 5 Effect of Smad6 on the transcriptional responses of TGF-β. **a**, Mink R mutant cells deficient in TβR-I were transfected with p3TP-Lux reporter, TβR-I and increasing amounts of Smad6 DNA. Cells were treated with (black bars) or without (white bars) 5 ng ml^{-1} TGF-β1 for 24 h. Smad6 inhibited luciferase activity induced by TGF-β in a dose-dependent manner. **b**, Smad6 also inhibited the activity of TβR-I (TD). **c**, Cyclin A luciferase reporter, pCAL2, was used to examine the effect of Smad6 on TGF-β signalling. TβR-I downregulated cyclin A luciferase activity, which was blocked by Smad6.

TβR-I (ref. 10). It was recently shown that TGF-β also induces association of Smad2 and Smad3 (ref. 13). We examined the effect of Smad6 on the heteromerization of these SMAD proteins. Smad2 formed a complex with Smad4 in the presence of constitutively active TβR-I, as shown by coprecipitation of Smad4 with Smad2. The complex formation was abolished by Smad6 (Fig. 4a, top). However, the interaction of Smad3 and Smad4 induced by TβR-I was not affected by Smad6 (Fig. 4b), consistent with the finding that Smad6 does not inhibit Smad3 phosphorylation (Fig. 3c). Furthermore, heteromerization of Smad2 and Smad3 was inhibited by Smad6 (Fig. 4c), suggesting that phosphorylation of both proteins is necessary for this interaction. These results suggest that Smad6 interferes specifically with the activation of Smad2 in TGF-β signalling.

We tested the role of Smad6 in TGF-β signalling in luciferase reporter gene assays. p3TP-Lux, a sensitive reporter for TGF-β, was used in R mutant mink cells deficient in TβR-I (Fig. 5). Wild-type TβR-I restored the TGF-β response in these cells. Smad6 suppressed the activation of the reporter gene in a dose-dependent manner (Fig. 5a) and also suppressed transcriptional activation by constitutively active TβR-I (Fig. 5b). Expression of cyclin A is necessary for cell-cycle progression and is suppressed by TGF-β[14]. Smad6 counteracted TGF-β in the cyclin A luciferase assay (Fig. 5c). These results indicate that Smad6 interferes with TGF-β signals in two distinct responses.

TGF-β is the prototype of a large family of cytokines that are involved in various biological activities[1–3]. Molecules related to TGF-β thus act in environments in which multiple signals interact and are likely to be under tight spatial and temporal regulation. SMAD proteins are required for signalling in the TGF-β superfamily, and could be targets for other forms of control inside the cell. Smad1, Smad2, Smad3 and Smad5 transduce ligand-specific signals, whereas Smad4 is an essential common partner of these ligand-specific SMAD proteins. Our results show that Smad6 belongs to a third class of the SMAD family. It has quite a different structure from the other SMAD proteins, and is likely to be a negative regulator of signalling by the TGF-β superfamily. □

Methods

Cloning of mouse Smad6 and northern blot analysis. A mouse lung cDNA library (Stratagene) was screened with an EST clone (429356) as a probe. One of the clones contained the entire coding region of mouse Smad6 and was sequenced using an ALFred sequencer (Pharmacia Biotech) and a Sequenase sequencing kit (USB). Sequence analysis was done with DNASTAR. Human and mouse tissue blots (Clontech) were probed with the EST clone.

Plasmids. Mammalian expression vectors with an N-terminal tag (Flag or Myc) were constructed by inserting oligonucleotides encoding the epitope-tag sequence into pcDNA3 (Invitrogen). The coding region of the mouse Smad6 was amplified by the polymerase chain reaction (PCR) and subcloned into Myc-pcDNA3 or Flag-pcDNA3. The integrity of the products was confirmed by sequencing. Smad1, Smad2, Smad3 and Smad4 expression plasmids were constructed in a similar manner.

Affinity crosslinking and immunoprecipitation. Iodination of TGF-β1 (R&D Systems), activin A (gift from Y. Eto) and OP-1/BMP-7 (gift from T. K. Sampath) and the following immunoprecipitation were performed as described[15].

Western blot and *in vivo* phosphorylation. COS-7 cells were transiently transfected using DMRIE-C (GibcoBRL). [^{32}P]orthophosphate- or [^{35}S]methionine/cysteine-labelling and immunoprecipitation were done as described[16]. For western blot analysis of the immunoprecipitated proteins, tagged proteins were detected by chemiluminescence (ECL, Amersham).

Luciferase assays. Mink R mutant cells were transiently transfected with an appropriate combination of a reporter, expression plasmids, and pcDNA3 using Tfx-50 (Promega). Total amounts of transfected DNA were the same in each experiment, and values were normalized using sea-pansy luciferase activity under the control of the thymidine kinase promoter (pRL-TK, Toyo, Ink).

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